

A method of interstrain differentiation of bacteria.**Summary of the invention**

5

The subject invention lies in the field of interstrain differentiation of bacteria. A general method has been developed with which various types of bacteria can be differentiated into separate individual strains. Thus in particular in the clinical setting this 10 method can suitably be used to determine what strain of bacterium is present in a sample. This new method is applicable for discerning between various strains of both Gram negative and Gram positive types of bacteria.

15

**Background of the invention**

20

Previously we had disclosed a method called oligotyping for interstrain differentiation of *Mycobacterium tuberculosis* strains in WO95/31569. It was stated in this document that one of the key factors in the control of tuberculosis is the rapid diagnosis of the disease and the 25 identification of the sources of infection. *M. tuberculosis* strain typing has already proved to be extremely useful in outbreak investigations (6, 14, 31) and is being applied to a variety of epidemiologic questions in numerous laboratories. Traditionally, laboratory diagnosis is done by microscopy, culturing of the micro-organism, skin testing and X-ray imaging. Unfortunately, these methods are often not sensitive, not specific and are very time-consuming, due to the slow growth rate of *M. tuberculosis*. Therefore, new techniques like in vitro amplification of *M. tuberculosis* DNA have been developed to rapidly detect the micro-organism 30 in clinical specimens (14). The ability to differentiate isolates of *M. tuberculosis* by DNA techniques has revolutionarized the potential to identify the sources of infection and to establish main routes of transmission and risk factors for acquiring tuberculosis by infection (1,3-10, 14, 16, 19-22, 25, 26, 27-33). The use of an effective universal 35 typing system will allow strains from different geographic areas to be compared and the movement of individual strains to be tracked. Such data may provide important insights and identify strains with particular problems such as high infectivity, high virulence and/or multidrug resistance. Analysis of large numbers of isolates may provide answers to 40 long-standing questions regarding the efficacy of BCG vaccination and the

frequency of reactivation versus reinfection.

The same problems identified for *M. tuberculosis* are inherent in differentiation of numerous other bacteria. The problems specifically arise for potentially epidemic pathogens and for bacteria that infect hospitals. A more rapid and simple typing method is required. Preferably the testing methods for various bacteria will occur in the same manner ensuring routine use for all types of bacteria for which testing is required. Preferably a test that can be carried out by non specialised personnel using little laboratory space and time is sought after.

The method disclosed in WO95/31569 is based on the DNA polymorphism found at a unique chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in *M. tuberculosis* complex bacteria. This locus was discovered by Hermans et al. (15) in *M. bovis* BCG, the strain used worldwide to vaccinate against tuberculosis. The DR region in *M. bovis* BCG consists of Directly repeated sequences of 36 base pairs, which are interspersed by non-repetitive DNA spacers, each 35 to 41 base pairs in length (15). The number of copies of the DR sequence in *M. bovis* BCG was determined to be 49. In other strains of the *M. tuberculosis* complex the number of DR elements was found to vary (15). The vast majority of the *M. tuberculosis* strains contain one or more IS6110 elements in the DR containing region of the genome.

It has been shown (12) that the genetic diversity in the DR region is generated by differences in the DR copy number, suggesting that homologous recombination between DR sequences may be a major driving force for the DR-associated DNA polymorphism (12). The high degree of DNA polymorphism within a relatively small part of the chromosome makes this region well-suited for a PCR-based fingerprinting technique.

Figure 1 depicts the structure of the DR region of *M. bovis* BCG as determined previously by Hermans et al. and Groenen et al. (12, 15). For the sake of convenience we will designate a DR plus its 3'adjacent spacer sequence as a "Direct Variant Repeat" (DVR). Thus, the DR region is composed of a discrete number of DVR's, each consisting of a constant part (DR) and a variable part (the spacer).

The method disclosed in WO95/31569 is based on a unique method of in vitro amplification of DNA sequences within the DR region and the hybridisation of the amplified DNA with multiple, short synthetic oligomeric DNA sequences based on the sequences of the unique spacer DNA's within the DR region (figure 2). This differs from previous PCR methods in the use of a set of primers with both primers having multiple

priming sites as opposed to having one of the primers bind to a fixed priming site such as to a part of IS6110. Because *M. tuberculosis* complex strains differ in the presence of these spacer sequences, strains can be differentiated by the different hybridisation patterns with a set of various spacer DNA sequences.

The method consists of in vitro amplification of nucleic acid using amplification primers in a manner known per se in amplification reactions such as PCR, LCR or NASBA, wherein a pair of primers is used comprising oligonucleotide sequences sufficiently complementary to a part of the Direct Repeat sequence of a microorganism belonging to the *M. tuberculosis* complex of microorganisms for hybridisation to a Direct Repeat to occur and subsequently elongation of the hybridized primer to take place, said primer being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction. Due to the multiple presence of Direct Repeats in the microorganisms to be detected the use of such primers implies that all the spacer regions will be amplified in an efficient manner. In particular it is not necessary for extremely long sequences to be produced in order to obtain amplification of spacers located at a distance from the primer. With the instant selection of the primer pairs a heterogenous product is obtained comprising fragments all comprising spacer region nucleic acid. Subsequently the detection of the amplified product can occur simply by using an oligonucleotide probe directed at one or more of the spacer regions one wishes to detect. In order to avoid hindrance in the amplification reactions the primers can have oligonucleotide sequences complementary to non-overlapping parts of the Direct Repeat sequence so that when both primers hybridize to the same Direct Repeat and undergo elongation they will not be hindered by each other. In particular to avoid any hindrance during elongation reactions when one primer DRa is capable of elongation in the 5' Direction and the other primer DRb is capable of elongation in the 3' Direction the DRa is selected such that it is complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary. The primer used must have an oligonucleotide sequence capable of annealing to the consensus sequence of the Direct Repeat in a manner sufficient for amplification to occur under the circumstances of the particular amplification reaction. A person skilled in the art of amplification reactions will have no difficulty in determining which length and which degree of homology is required for good amplification

reactions to occur. The consensus sequence of the Direct Repeat of microorganisms belonging to the *M. tuberculosis* complex is given in sequence id. no. 2 and in figure 1.

In addition to what has already been disclosed in W095/31569 we 5 also determined the spoligotypes of *M. tuberculosis* strains which were subcultured for many months both in the laboratory and in guinea pigs. The strains selected for this purpose were those used in a previous study on the stability of IS 6110 (2). All subcultured strains displayed the identical spoligotype patterns compared with the primary cultures thus 10 indicating the pace of the molecular clock in this instance is slow enough for use in epidemiology of the disease.

Because of the large success and simplicity of the method for Mycobacterium tuberculosis strain differentiation and in view of problems 15 in strain differentiation with other microorganisms we used the Direct Repeat consensus sequence to screen data bases with nucleic acid sequences from other microorganisms. Unfortunately no further matches were found. The Direct Repeat sequence appeared to be unique for the Mycobacterium tuberculosis as did their spacer sequences. As to date no 20 function had actually been attributed to the Direct Repeat sequence it was unexpected that the sequence was universally distributed among other types of microorganisms. Such would at best be expected if the sequence had a function that was required also in other organisms.

#### Description of the invention

25 Notwithstanding the negative result after screening with the Direct Repeat consensus sequence we considered further analysis of known sequences by looking for a pattern in the nucleic acid sequences of other microorganisms reminiscent of the Direct Repeat-spacer pattern in 30 Mycobacterium tuberculosis. Quite unexpectedly we found using a specifically designed computer programme that such patterns existed in a large number of other microorganisms with a broad range of genera. It appears that the DR-like sequences are very common in prokaryotes. They are however noticeably absent in eukaryotes. Chapter III of Bergeys 35 Determinative Manual of Bacteriology Ninth edition (11) provides a table of characteristics for distinguishing prokaryotes from eukaryotes i.e. distinguish bacterium from microscopic eukaryotes in the shape of mold, yeast, algae or protozoans.

All bacterial sequences analysed revealed the presence of such

a sequence structure and thus the oligotyping method illustrated for *Mycobacterium tuberculosis* can be applied for differentiating between all strains of bacteria. It was totally unexpected that a consensus structure of this type could be universally found. The Direct Repeat sequences themselves are different between different genera but the general framework of a cluster of Direct Repeat sequences, separated by a number of non repetitive spacers is universally present in bacterial genomes. Considering the fact that thusfar no function has been attributed to such a region in *Mycobacterium tuberculosis* or in fact for any of the sequences comprising Direct Repeat like regions in any other bacteria for which such sequences had been described this is remarkable.

Bacteria can be divided into Archaebacteria and Eubacteria. The eubacteria in turn can be distinguished into Gram-negative and Gram-positive bacteria with cell walls and Eubacteria lacking cell walls. Chapter IV of Bergeys determinative Manual of Bacteriology Ninth edition (11) reveals the characteristics for each group. Over a wide range of the subgroups in these 4 groups we have found the presence of the consensus structure i.e. the presence of DR-like loci. The IV groups have been subdivided by Bergey into more than 30 subgroups. We have examples in Groups 3,4,5 and 6, Group 11, 17, 31, 32, 33. The method according to the invention is particularly of interest for the bacteria that are pathogenic for humans. Group 4 comprises Gram negative bacteria. Genera from Group 4 are *Legionella* (which can cause pneumonia) and Legionnaires disease, the genus *Neisseria* (of which *Neisseria meningitidis* is well known as causative agent of meningitis and of which *Neisseria gonorrhoeae* is another example), the genus *Pseudomonas* (renown for hospital infections) and the genus *Bordetella* (of which *Bordetella pertussis* is well known as causative agent of whooping cough). In Group 5 bacteria as defined in Bergeys Manual the Enterobacteriaceae form a family of 30 genera. These bacteria form a particularly interesting group of Gram negative bacteria that infect humans. Suitable examples of genera from this family are *Enterobacter*, *Escherichia*, *Shigella*, *Salmonella*, *Serratia*, *Klebsiella* and *Yersinia*. Other less well known pathogenic Enterobacteriaceae genera are *Cedeca*, *Citrobacter*, *Kluyvera*, *Leclercia*, *Pantoea*, *Proteus*, *Providencia* and *Hafnia*. Other Group 5 families are *Pasteurellaceae* with the genus *Haemophilus* and the family *Vibrionaceae* with the genus *Vibrio*. *Haemophilus influenzae* is a leading cause of meningitis in children and also other septicemia conditions. *Vibrio cholerae* is the causative agent of cholera, *V. parahaemolyticus* can cause

food poisoning and *V. vulnificus* causes highly fatal septicemia.

Of the Enterobacteriaceae *Shigella*, *Escherichia* and *Salmonella* are best known and difficult to differentiate. *Shigella* is an intestinal pathogen of humans causing bacillary dysentery. Well known strains are *S. dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei*. The genus *Salmonella* is well known for food poisoning. Well known *Salmonella* strains are *S. typhimurium*, *S. arizona*, *S. choleraesuis*, *S. bongori*. *Salmonella* are also causative agents of typhoid fever, enteric fevers, gastroenteritis and septicemia. The genus *Serratia* bacteria are opportunistic pathogens for hospitalized humans causing septicemia and urinary tract infections. Examples are *S. liquefaciens* and *S. marcescens*. Of the *Escherichia* *E. coli* is best known as major cause of urinary tract infections and nosocomial infections including septicemia and meningitis. Other species are usually associated with wound infections.

Enterobacter constitutes a problem genus of opportunistic pathogens causing burn wound and urinary tract infections occasionally also meningitis and septicemia. Well known species are *E. cloacae*, *E. sakazakii*, *E. aerogenes*, *E. agglomerans*, *E. gergoviae*. *Klebsiella* are also causative agents of bactriemia, pneumonia, urinary tract and other human infections in urological, neonatal, intensive care and geriatric patients. *Klebsiella pneumoniae* and *K. oxytoca* are examples of species in the genus.

Particularly interesting from a clinical point of view are also the Gram positive pathogenic bacteria. The genera *Streptococcus* and *Staphylococcus* form examples of such bacteria. *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus* are examples thereof. Of the mentioned groups and genera the pathogenic bacteria are of interest. These bacteria are dangerous when infecting hospitals in particular.

Due to the increasing incidence of infection differentiation of potentially epidemiological organisms is also of interest. Such organisms comprise *Bordetella pertussis* and *Neisseria meningitidis* the causative organism of meningitis is of particular interest. Quite specifically pathogenic bacteria infecting hospitals and bacteria capable of causing epidemics are targets for the differentiation method according to the invention.

The invention consists of a method of in vitro amplification of nucleic acid using amplification primers in a manner known per se, in amplification reactions such as PCR, LCR or NASBA, wherein a pair of primers is used comprising oligonucleotide sequences sufficiently

complementary to a part of the Direct Repeat sequence of a bacterium other than a microorganism belonging to the *M. tuberculosis* complex of microorganisms for hybridisation to a Direct Repeat to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction, wherein the Direct Repeat is a sequence with a length between 20-50 base pairs which occurs 5-60 times in a contiguous region of the bacterial genome, whereby the Direct Repeat sequences are separated by spacer sequences with a length of between 20-50 nucleotides, said spacer sequences being non repetitive. By using the programme Patscan e.g. on the nucleic acid data bases for microorganism genomic sequences such motifs and thus also the identities of the various species specific Direct Repeats and the corresponding spacer sequences can be obtained. In the Patscan programme the Direct Repeat can be designated p1 with a length between 20-50 basepairs then search for p1 20-50 basepairs downstream of p1. Thus this pattern in Patscan is described as  $p1=(20..50)(20..50)p1(20..50)p1$ . The length of the sequences can be varied as can the intermediate distance and the number of times the Direct Repeat has to occur. A Direct Repeat can often have a length of 30-40 base pairs with a spacer length of 35-45 base pairs. Basically we looked for a stretch of identical repeat sequences interspersed by spacer sequences which do not necessarily share much of their sequence with the Direct Repeat of *M. tuberculosis*. The patscan programme is freely accessible at the Internet site:<http://www-c.mcs.anl.gov/home/overbeek/-PatScan/HTML/patscan.html>. The programme was written by Ross Overbeek Mathematics and Computer Science Division Argonne National Laboratory Building 221 Room D-236 9700 S. Cass Avenue Argonne IL 60439 USA.

Most of the Repeats exhibit one or more of the following characteristics, they end with a sequence similar to GAAAC i.e. exhibit at least 3 of the nucleotides of this consensus sequence at the terminus, preferably 4 or 5, start with CTTTG, have stretches of 3-4 identical bases. The termini can for example be selected from GAAAC, GAAXC GAACTC, GXAAC, GCAAC, GAAA, GAAXC, GAAGC and AAAC. Suitable Termini are provided in Table II.

Organisms as diverse as the Archaeabacteria e.g. *Methanococcus jannasschi* (Group 31), *Haloferax mediterranei* (Group 33), the cyanobacteria *Calotrix* (Group 11), and *Anabeana* (Group 11), and purple bacteria e.g. *E.coli* (Group 5), *Mycobacterium tuberculosis* (Group 21) and

Thermus thermophilus (Group 4), Archaeoglobus (Group 32) and Thermotoga (Group 6) were found to possess DR-like sequences upon analysis of their genomes using the PatScan programme. In the subsequent study of literature from which these data were derived it also became clear from 5 Southern blots that the Repeat sequences were also found in related species.

With regard to the genetic organisation the structures of the DR-like loci in the microorganisms is rather variable (figure 3). In *M. tuberculosis* the DR locus is large and in most isolates it is disrupted 10 by an insertion element. This is also the case in *T. thermophilus*, however here the number of DVR's is only 11 and the DR locus is disrupted by two insertion elements. In *E. coli* K12 2 DR loci are present separated by approximately 22kb; in *Anabaena* the locus is of intermediate size and interrupted by a 130 bp sequence of unknown function or origin. In *H. 15 mediterranei* the DR locus is of intermediate size and not disrupted, however there is evidence for a second DR locus on one of the mega plasmids found in this organism. In *M. jannaschii* there is one locus of intermediate size but at several other positions in the genome one or a few other DVR's are found. In most cases the DVR's are linked to a so-called Long Repeat (LR) element of unknown function. Also in *M. jannaschii* mega plasmids are found but in contrast to *H. mediterranei* 20 they do not contain DR sequences.

Accession numbers for the sequences of various organisms for 25 which the DR like loci have been found are provided here. For *E. coli* and *Shigella* M27059, M27060, U29579, U29580 and M18270. The relevant portions of the sequences are also disclosed by Blattner for *E. coli*. Nakata et al reveal in the Journal of Bacteriology (13) that downstream of the iap region a sequence of 29 bases appears 14 times 32 or 33 base pairs apart. Nucleotide sequences hybridizing to the 29 base pair sequence were also 30 detected in *Shigella dysenteriae* and *Salmonella typhimurium*.

A DR-like sequence was found in the contig 214 of *S. pyogenes* M1(ATCC 700294) of the genome sequencing project of the University of Oklahoma. Further research into this DR-like sequence in other *S. pyogenes* revealed spacer polymorphism. The DR regions of eight *S. pyogenes* isolates were studied. The DR regions were isolated by PCR using 35 primers that were derived from the database (University of Oklahoma, serotype M1 ATCC 700294. The sequence data is available under <http://www.genome.ou.edu>. This strain contains seven repeats and six spacers.

7/10/02

Five of the isolates gave a PCR product, these were a M2 strain, a M4 strain and three M1 strains. The M4 strain contained only a single repeat sequence that was flanked by the same sequences as the ATCC 700294. The M2 strain sequencing did not work, but the size of the PCR fragment indicated that two repeats are present. The three M1 strains were all the same, they contained four repeats and three spacers. The repeats were identical to ATCC 700294, while one of the spacers was identical to ATCC 700294 and two were different.

These studies on *S. pyogenes* show that the DR regions have conserved spacers and repeat sequences.

The *Salmonella* genomic sequence as sequenced by the University of Washington St Louis has also revealed the presence of DR-like sequences. The DR exhibits high homology with the Direct Repeat of *E. coli*. One of the contigs revealed 7 Repeats and 6 spacers.

A panel of five *E. coli* isolates and three *Shigella* strains were studied. The five *E. coli* isolates were selected to have an optimal diversity, they were isolated from different species or geographic regions. The *Shigella* strains are considered separate (sub)species. See Table 1. The isolates were obtained from the collection of Dr. Wim Gaastra.

Table 1

species	description	DRI*	DRII*
<i>E. coli</i>	184	American isolate	Southern
	358	human urinary tract	Southern
	968	mastitis	Southern
	1008	chicken	PCR
	1732	human intestine	Southern
<i>Shigella disenteriae</i>	593		PCR
	sonnei		Southern
	boydii		PCR

\* The DR regions were identified by Southern blot of genomic DNA and DRI and DRII regions of *E. coli* K12. When PCR is indicated the DR regions were identified by the Southern and the PCR. This PCR was done with primers derived from the K12 sequence.

The DRI and DRII sequences that could be amplified by PCR were

cloned and sequenced. Somehow the DRI regions could not be amplified by PCR using the primers designed on the K12 sequence, while the Southern data demonstrate that DRI is present. Apparently, the recognitions sites for the primers are polymorphic. The sizes of the DRRII regions were found to vary greatly between these isolates. The smallest was a single repeat in the *S. sonnei* strain and the largest was a repeat cluster of at least 15 repeats in *E. coli* isolate 1008. The sequences of the repeats were highly conserved between these isolates. The *S. typhimurium* data is obtainable from the Internet <http://genome.wustl.edu/gsc/bacterial/salmonella.html>.

The spacer sequences almost all were unique. Approximately 40 spacers have been sequenced and only three of them were already known from a previously sequenced DR region. This indicates a high number of different spacer sequences in *E. coli*.

Accession number X73453 provides the Halerofax mediterranei sequence. The sequence can also be found in Molecular Microbiology 17 of 1995 in an article by Mojica et al. (17). The Repeat sequence has also been found in related species.

The genomic project of the Methanococcus jannaschii reveals a DR-like sequence as is apparent from the Bult et al article in Science 273 of 1996 (18). The Accession number is U67459 i.a.

Accession number X87270 for Anabeana sp reveals 17 spacers and a LTRR element. These elements also occur in related species of cyanobacteria such as Calotrix. The sequence data are provided by Masepohl et al in BBA 1307 1996 (23).

Accession number AE000782 for Archaeoglobus fulgidus reveals three DR-like Repeats with the same Repeat sequence and the this has a slightly larger but closely related Repeat. The Repeats are present 20-30 times. The spacers are unique sequences. H.P. Klenk discloses sequence data in Nature 390 1997 (24).

The invention also covers a method of detection of a bacterium, said bacterium not belonging to the *M. tuberculosis* complex of microorganisms said method comprising

- 1) amplifying nucleic acid from a sample with the amplification method according to any of the preceding described embodiments of the amplification method according to the invention, followed by
- 2) carrying out a hybridisation test in a manner known per se, wherein the amplification product is hybridised to an oligonucleotide probe or a plurality of different oligonucleotide probes, each

oligonucleotide being sufficiently homologous to a part of a spacer of the Direct Region of the bacterium to be determined for hybridisation to occur to amplified product if such spacer nucleic acid was present in the sample prior to amplification, said hybridisation step optionally being carried out without prior electrophoresis or separation of the amplified product.

5 3) detecting any hybridised products in a manner known per se.

The method can be carried out in a manner such that the hybridisation test is carried out using a number of oligonucleotide probes, said number comprising at least a number of oligonucleotides probes specific for the total spectrum of bacteria it is desired to detect. In a suitable embodiment of a method according to the invention the oligonucleotide probe is at least seven oligonucleotides long and is a sequence complementary to a sequence selected from any of the spacer sequences of the Direct Repeat region of the bacterium to be determined or is a sequence complementary to fragments or derivatives of said spacer sequences, said oligonucleotide probe being capable of hybridising to such a spacer sequence and comprising at least seven consecutive nucleotides homologous to such a spacer sequence and/or exhibiting at least 60% homology, preferably exhibiting at least 80% homology with such a spacer sequence.

Preferably the method according to the invention is carried out to determine the presence and nature of a pathogenic bacterium selected from the group of Gram negative bacteria of Groups 4 and 5 of Bergeys Determinative Manual of Bacteriology ninth edition. Of particular interest due to damage caused by such pathogens are bacteria belonging to the families Enterobacteriaceae, Pasteurellaceae and Vibrionaceae of Group 5, most specifically the Enterobacteriaceae. Also of interest are the Gram positive bacteria of Group 17. Suitable examples of genera of the pathogenic bacterium to be detected from the group of Gram negative bacteria of Bergeys Determinative Manual of Bacteriology ninth edition are Escherichia, Shigella, Salmonella, Klebsiella, Enterobacter, Yersinia, Serratia, Haemophilus, Vibrio, Legionella, Neisseria, Pseudomonas and Bordetella. For the group of Gram positive bacterial genera Staphylococcus and Streptococcus are targets for the differentiation method.

Suitably in a method according to the invention for differentiating the type of bacterium in a sample, said bacterium not belonging to the M. tuberculosis complex the hybridisation pattern is



Table II

Species	Repeat sequence	Reference	EMBL/Genbank accession number
<i>Mycobacterium tuberculosis</i> <i>Escherichia coli</i>	GTCGTAGACCCAAAACCCCGAGGGGACGGAAAC CGGGTTATCCCCGCTGGCGGGAACTC	Hermans et al. Nakata et al. J.Bact. 171 3553-3556 (1989)	
<i>Shigella dysenteriae</i>	CGGGTTATCCCCGCTGGCGGGAACTC	our own data	M27059 and M27060
<i>Shigella sonnei</i>	CGGGTTATCCCCGCTGGCGGGAACTC	our own data	
<i>Shigella boydii</i>	CGGGTTATCCCCGCTGGCGGGAACTC	our own data	
<i>Salmonella enteritidis</i>	CGGGTTATCCCCGCTGGCGGGAACTC	our own data	
<i>Seratia marcescens</i>	CGGGTTATCCCCGCTGGCGGGAACTC	our own data	
<i>Salmonella typhimurium</i>	CGGGTTATCCCCGCTGGCGGGATAACAC	contig 70A06 of the typhimurium genome project. Univ. of Washington St. Louis	
<i>Streptococcus pyogenes</i>	GTTTAGAGCTATGCTGTTGATGGTCCAAACT	Contig 214 of the S. pyogenes genome project University of Oklahoma	

(Table II)

Species	Repeat sequence	Reference	EMBL/Genbank accession number
<i>Thermus aquaticus thermophilus</i>	AATCCCTTACGGGCTCAATCCCTTGCAA	Ashby et al. Plasmid 24 1-11 (1990) Liebl. et al. Microbiol. 142 2533-2542 (1996)	M33159 Z69341
<i>Thermotoga maritima</i>	GTTCATAACTTCCCTAGAGGTATGGAAC	Masepohl BBA 1307 26-30 1996 idem	X87270 Z47161
<i>Anabaena</i>	GTTTAACTAACAAAAATCCCTATCAGGGATTGAAAC	Mojica et al. Molec. Microbiol. 17 85-93 (1995)	X7453
<i>Calotrix</i>	GTTTAACTTATAAAAATCCCTTAGGGATTGAAAC	Bult et al. Science 273 1058-1073 (1996)	U67459 (for the large DR-like region)
<i>Halofenax mediterranei</i>	GTACAGACGAACCCATAGTTGGGTGAAGC	Smith et al. J. Bact. 179 7135-7155 (1997)	AE000920 (for R2)
<i>Methanococcus jannaschii</i>	AATTAAATCAGACCGTTGGAAATGGAAA	ATTCAATCCCATTGGTCTGATTAAAC	AE000878 (for R1)
<i>Methanobacterium</i>			AE000782 (whole genome)
<i>thermoautotrophicum</i>	GTAAAATCAGACCAAATGGGATTGAAT		
<i>Archaeoglobus fulgidus</i>	CTTCAATCCCATTGGTCTGATTAAAC		

Not only the above methods fall within the scope of the invention but also specifically selected primer pairs for carrying out such a method. A pair of primers according to the invention is a pair wherein both primers comprise oligonucleotide sequences of at least 7 oligonucleotides and are sufficiently complementary to a part of the Direct Repeat sequence of the microorganism *E. coli* for hybridisation to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 10 3' Direction and wherein sufficiently complementary means said oligonucleotide sequence comprises at least seven consecutive nucleotides homologous to such a Direct Repeat sequence and/or exhibits at least 60% homology, preferably at least 80% homology, most preferably more than 90% homology with the corresponding part of the Direct Repeat sequence. 15 Suitable Direct Repeat sequences are provided in Table II. In particular such a primer pair can comprise one primer DRa capable of elongation in the 5' Direction and the other primer DRb capable of elongation in the 3' Direction with DRa being complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary, the Direct Repeat being present in the Direct Region of 20 *E. coli*. Another suitable pair comprises primers with oligonucleotide sequences of at least 7 oligonucleotides and are sufficiently complementary to a part of the Direct Repeat sequence of the microorganism *S. typhimurium* for hybridisation to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction and wherein sufficiently complementary means said oligonucleotide sequence comprises at least seven consecutive nucleotides homologous to such a 25 Direct Repeat sequence in particular the Sequence provided in Table II and/or exhibits at least 60% homology, preferably at least 80% homology, most preferably more than 90% homology with the corresponding part of the Direct Repeat sequence. In particular such a pair comprises one primer DRa capable of elongation in the 5' Direction and the other primer DRb capable of elongation in the 3' Direction with DRa being complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary, the Direct Repeat being 30 present in the Direct Region of *S. typhimurium*. 35

Kits for carrying out a differentiation method according to any

of the described embodiments also fall within the scope of the invention. Such kits comprise a primer pair according to any of the described embodiments and optionally an oligonucleotide probe or a carrier, said carrier comprising at least 1 oligonucleotide probe specific for a spacer 5 region of a bacterium to be determined said bacterium not belonging to *M. tuberculosis* complex, preferably the oligonucleotide probe as defined, being an oligonucleotide probe of at least 10 nucleotides, preferably more than 12 nucleotides, in particular comprising between 12 to 40 nucleotides, said probe being sufficiently homologous to any of the 10 spacer sequences or to fragments or derivatives of such spacer sequences to hybridise to such a spacer sequence, said oligonucleotide probe comprising at least 10 consecutive nucleotides homologous to such a spacer sequence and/or exhibiting at least 60% homology, preferably exhibiting at least 80% homology, most preferably exhibiting more than 15 90% homology with the corresponding part of the spacer sequence. Suitably a kit according to the invention comprises a data carrier with required reference patterns of the bacterial strain to be determined.

20

## REFERENCES

1. Beck-Sagué, C., S.W. Dooley, M. D. Hutton, J. Otten, A. Breeden, J.T. Crawford, A.E. Pitchenik, C. Woodley, G. Cauthen, W.R. Jarvis. 1992 Hospital outbreak of multidrug-resistant *Mycobacterium tuberculosis* infections. *JAMA*. 268: 1280-1286.
2. Van Soolingen, D., P.W.M. Hermans, P.E.W. de Haas, D. R. Soll, and J.D.A. van Embden. 1991. The occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains; evaluation of IS-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J. Clin. Microbiol.* 29:2578-2586.
3. CDC. 1992. Transmission of multidrug-resistant tuberculosis among immunocompromised persons in a correctional system. New York 1991. *MMWR* 41: 507-509.
4. CDC. 1991. Nosocomial transmission of multidrug resistant tuberculosis among HIV-infected persons, Florida and New York, 1988-1991. *MMWR* 40: 585-591.
5. Cave, M.D., K.D. Eisenach, P.F. McDermott, J.H. Bates, and J.T.

Crawford. 1991. IS6110: Conservation of sequence in the *Mycobacterium tuberculosis* complex and its utilization in DNA fingerprinting. *Mol. Cell. Probes* 5:73-80.

6. Daley, C.L. P.M. Small, G.F. Schecter, G.K. Schoolnik, R.A. McAdam, W.R. Jacobs, Jr., and P.C. Hopewell. 1992. An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus: an analysis using restriction fragment length polymorphisms. *N. Engl. J. Med.* 326:231-235.

10 7. Dooley, S.W., M.E. Villarino, M. Lawrence, et al. 1992. Nosocomial transmission of tuberculosis in a hospital unit for HIV-infected patients. *JAMA*. 267:2632-2634.

8. Dwyer, B., K. Jackson, K. Raios, A. Sievers, E. Wilshire and B. Ross. 1993. DNA Restriction fragment analysis to define an extended cluster of tuberculosis in homeless men and their associates. *J. Inf. Diseases*, 167: 490-494.

15 9. Edlin, B.R., J.I. Tokars, M.H. Griebo, J.T. Crawford, J. Williams, E.M. Sordillo, K.R. Ong, J.O. Kilburn, S.W. Dooley, K.G. Castro, W.R. Jarvis and S.D. Holmberg. 1992. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. *N. Eng. J. Med.* 326:1514-1521.

20 10. Fischl, M.A, R.B. Uttamchandani, G.L. Daikos, et al. 1992. An outbreak of tuberculosis caused by multiple-drug resistant tubercle bacilli among patients with HIV infection. *Ann. Intern. Med.* 117:177-183.

25 11. Bergeys Determinative Manual of Bacteriology Ninth Edition

12. Groenen, P.M.A., A.E. van Bunschoten, D. van Soolingen, and J.D.A. van Embden. 1993. Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; Application for strain differentiation by a novel method. *Mol. Microbiol.* (1993) 10 (5) 1057-1065.

30 13. Nakata et al Journal of Bacteriology June 1989 p3553-3556.

14. Hermans, P.W.M., D. van Soolingen, J.W. Dale, A.R. Schuitema, R.A. McAdam, D. Catty, and J.D.A. van Embden. 1990. Insertion element IS986 from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. *J. Clin. Microbiol.* 28:2051-2058.

35 15. Hermans, P.W.M., D. van Soolingen, E.M. Bik, P.E.W. de Haas,

J.W. Dale, and J.D.A. van Embden. 1991. The insertion element IS987 from *M. bovis* BCG is located in a hot spot integration region for insertion elements in *M. tuberculosis* complex strains. *Infect. Immun.* 59:2695-2705.

5 16. Hermans, P.W.M., D. van Soolingen, and J.D.A. van Embden. 1992. Characterization of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of *Mycobacterium kansasii* and *Mycobacterium gordonae*. *J. Bacteriol.* 174:4157-4165.

10 17. Mojica et al., *Molecular Microbiology* 17, 1995

18. Bult et al., *Science* 273, 1996

19. Mazurek, G.H., M.D. Cave, K.D. Eisenach, R.J. Wallace JR, J.H. Bates, and J.T. Crawford. 1991. Chromosomal DNA fingerprint patterns produced with IS6110 as strain specific markers for epidemiologic study of tuberculosis. *J. Clin. Microbiol.* 29:2030-2033.

15 20. McAdam, R.A., P.W.M. Hermans, D. van Soolingen, Z.F. Zainuddin, D. Catty, J.D.A. van Embden, and J.W. Dale. 1990. Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS3 family. *Mol. Microbiol.* 4:1607-1613.

20 21. Mendiola, M.V., C. Martin, I. Otal, and B. Gicquel. 1992. Analysis of regions responsible for IS6110 RFLP in a single *Mycobacterium tuberculosis* strain. *Res. Microbiol.* 143:767-772.

25 22. Otal, I., C. Martin, V. Vincent-Lévy-Frébault, D. Thierry, and B. Gicquel. 1991. Restriction fragment length polymorphism analysis using IS6110 as an epidemiological marker in tuberculosis. *J. Clin. Microbiol.* 29:1252-1254.

23. Masepohl et al., kBBA 1307 1996

30 24. Klenk, H.P., *Nature* 390, 1997

25. Palittapongarnpim, P.S., S. Rienthong, and W. Panbangred. 1993. Comparison of restriction fragment length polymorphism of *M. tuberculosis* isolated from cerebrospinal fluid and sputum: a preliminary report. *Tubercle and lung disease* 1993. 74, 204-207.

35 26. Ross, C., K. Raios, K. Jackson, and B. Dwyer. 1992. Molecular cloning of a highly repeated element from *Mycobacterium tuberculosis* and its use as an epidemiological tool. *J. Clin. Microbiol.* 30:942-946.

27. Small, P.M., R.W. Schafer, P.C. Hopewell, S.P. Singh, M.J. Murphy, E. Desmond, M.F. Sierra, and G.K. Schoolnik. 1993. Exogenous reinfection with multidrug-resistant *M. tuberculosis* in patients with advanced HIV infection. *N. Eng. J. Med.* 328:1137-1144.

5 28. Thierry, D., M.D. Cave, K.D. Eisenach, J.T. Crawford, J.H. Bates, B. Gicquel, and J.L. Guesdon. 1990. IS610, an IS-like element of *M. tuberculosis* complex. *Nucleic Acids Res.* 18:188.

10 29. Van Embden, J.D.A., D. van Soolingen, P.M. Small, and P.W.M. Hermans. 1992. Genetic markers for the epidemiology of tuberculosis. *Res. Microbiol.* 143: 385-391.

15 30. Van Embden, J.D.A., M.D. Cave, J.T. Crawford, J.W. Dale, K.D. Eisenach, B. Gicquel, P.W.M. Hermans, C. Martin, R. McAdam, T.M. Shinnick, and P.M. Small. 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting; Recommendations for a standardized Methodology *J. Clin. Microbiol.* 31:406-409.

20 31. Van Soolingen, D., P.W.M. Hermans, P.E.W. de Haas, and J.D.A. van Embden. 1992. Insertion element IS1081-associated Restriction Fragment Length Polymorphism in *Mycobacterium tuberculosis* Complex spacies: a reliable tool for recognizing *Mycobacterium bovis* BCG. *J. Clin. Microbiol.* 30:1772-1777.

25 32. Van Soolingen, D., P.E.W. de Haas, P.W.M. Hermans, P.M.A. Groenen and J.D.A. van Embden. 1993. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 31:1987-1995.

30 33. Yuen L.K., B.C. Ross, K.M. Jackson, and B. Dwyer. 1992. Characterization of *Mycobacterium tuberculosis* strains from Vietnamese patients by southern blot hybridization. *J. Clin. Micro.* 31: 1615-1618.

35 34. Kamerbeek J., L.M. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, J.E. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal and J.D.A. van Embden 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J. Clin. Microbiol.* 35: 907-914.

## DESCRIPTION OF THE FIGURES

Figure 1 depicts the structure of the DR region of *M. bovis* BCG as determined previously by Hermans et al. and Groenen et al. (12, 15). For the sake of convenience we will designate a DR plus its 3'adjacent spacer sequence as a "Direct Variant Repeat" (DVR). Thus, the DR region is composed of a discrete number of DVR's, each consisting of a constant part (DR) and a variable part (the spacer).

Figure 2 depicts multiple, short synthetic oligomeric DNA sequences based on the sequences of the unique spacer DNA's within the DR region.

Figure 3 shows the genetic organisation of the structures of the DR-like loci in various bacterial species.

► depicts the transcription direction of open reading frame (ORF)

For *M. tuberculosis*: MTCY 16B7.26, 27 and 30C are unknown genes/proteins.

For *E. coli*: iap gene function is alkaline phosphatase isozyme conversion. ORF f94, f305, YGCE and f223 are unknown genes/proteins.

For *S. pyogenes*: ORF1 and 2 are unknown genes/proteins.

For *T. thermophilus*: ORFC and D are unknown genes/proteins and ORF 1A and 1B are possibly transposases of IS elements 1000 and 1000A.

For *Anabaena*: No ORFs were annotated in the flanking sequences. The 130 bp insert is of unknown origin.

For *Haloferax mediterranei*: ORF21 is an unknown gene/protein. Probably another repeat cluster is also present on the megaplasmid pHM500.

For *Methanococcus jannaschii*: Comprises about 10 repeat clusters, the largest one of which comprises 25 repeats. All repeat clusters are coupled to a Long Repeat (LR) segment of 425bp. There are 18 LR's, some of which contain only one repeat. Smaller LR segments are also present, ALR. In one case, a cluster contains 5 repeats without LR (see ref. 18)

For *M. thermoautrophicum*: Two repeat clusters SRI and SRII flanked by

LRI, LRII. LRI and LRII are almost identical and are homologues of the LR segment of *M. jannaschii*. SRI and SRII are separated by 500 kb in the genome.

5 For *Thermatoga maritima*:CelA gene encodes cellulase: endo-1,4-beta-glucanase (EC 3.2.1.4) and CelB is also a cellulase exhibiting 58% identity with celA.

10 For *Archaeoglobus fulgidus*: The SRIA and SRIB repeat clusters have the same Repeat Sequence and the SRII Repeat Sequence is also clearly homologous. The SR clusters are separated by about 400bp. SRIB and SRII are located near tRNA genes. SRIA lies adjacent to an unknown ORF3.

Figure 4

15 Hybridization Patterns of 17 *E. coli* isolates. Thirty four different spacer oligonucleotides were covalently linked to a membrane and PCR amplified DNA of *E. coli* was hybridized as described (Kamerbeek et al. 1997), except that the primers used to amplify the DR locus were specific for the DR sequence from *E. coli*. Note the polymorphism observed in *E. coli* due to the strain-dependent presence or absence of spacer DNA.

20 Figure 5

25 Hybridization Patterns of 4 *Salmonella typhimurium* isolates. Six different spacer oligonucleotides were covalently linked to a membrane and PCR amplified *Salmonella* DNA was hybridized as described (Kamerbeek et al 1997), except that the primers used to amplify the DR locus were specific for the DR locus of *E. coli*. Note the polymorphism observed in *Salmonella* due to the strain-dependent presence or absence of spacer DNA.